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Polyacrylamide-Gel Electrophoresis of Soybean Whey Proteins and Trypsin Inhibitors

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Polyacrylamide-gel electrophoresis with glycine buffer (pH 9.2) containing 8 M urea separated soybean whey proteins into at least 24 bands. In contrast, ultracentrifugation indicated only 2 fractions; moving-boundary electrophoresis, 8–9 components; and column chromatography, 13 or more proteins. A prominent, fast-moving band in the gel pattern appeared to be identical to crystalline soybean trypsin inhibitor. Examination of 9 commercial samples of trypsin inhibitor showed multiple bands in all; most samples separated into 6 or more bands, and one preparation resolved into at least 13 bands in the gel. An inhibitor sample isolated by column chromatography and apparently identical to crystalline inhibitor also appeared heterogeneous although it contained fewer minor bands than the commercial samples. Three other trypsin inhibitor fractions recently isolated by column chromatography likewise yielded multiple bands but distinctly different from crystalline soybean trypsin inhibitor.

Polyacrylamide-gel electrophoresis appears to be a sensitive tool for examining soybean whey protein fractions and should greatly facilitate future fractionation studies on this complex protein mixture.

Moving-boundary electrophoresis has been of limited usefulness for characterizing soybean proteins. Briggs and Mann (1), the first to use moving-boundary electrophoresis for analysis of soybean proteins, reported partial separation of the water-soluble proteins into seven or more components. Kondo *et al.* (2) reported at least five components in a 1.5 N sodium chloride extract of soybean meal, and Smith *et al.* (3) in 1955 reported a minimum of eight peaks in the acid-precipitable and whey proteins of the soybean. In the latter study the best resolution was obtained with the whey protein fraction.

Since then ultracentrifugation (4) and column chromatography (5, 6) have shown more clearly the complexity of soybean proteins, but both methods have their limitations. For example, Rackis *et al.* (5) and Morrison (7) showed the presence of 13 or more components in soybean whey proteins by column chromatography. One component isolated was soybean trypsin inhibitor (SBTI). Later, Rackis *et al.* (8, 9) reported chromatographic isolation of three other SBTI's.

Studies by Roberts (10) indicate that polyacrylamide-gel electrophoresis may be a useful analytical tool for characterizing soybean proteins. We have examined gel electrophoresis in detail to find conditions for analyzing soybean whey proteins. Here we describe our results on the whey proteins and SBTI's, which are major constituents of the whey protein mixture.

EXPERIMENTAL METHODS

Preparation of soybean proteins. Undenatured hexane-defatted soybean flakes, laboratory prepared, were twice extracted with water, first at a

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solvent-to-meal ratio of 10:1 for 1 hour, then a second time at a 5:1 ratio for 15 minutes. After centrifugation the two extracts were combined. A portion of the combined extract was removed and dialyzed at 2° against phosphate buffer pH 7.6 (μ = 0.5 with 0.01 M 2-mercaptoethanol (11)) for 2 days, then against water for 10 days, and freeze-dried to yield water-extractable soybean proteins.

The remainder of the extract was adjusted to pH 4.4 with 1 N HCl; the precipitate was collected by centrifugation, washed three times with water, and freeze-dried to give the acid-precipitated protein.

The supernatant solution at pH 4.4 (whey) was brought to pH 8 with NaOH and allowed to stand 1 hour at 4° to precipitate phytate salts, which were removed by centrifugation; the solubles were then dialyzed against water 3–4 days and freeze-dried to yield whey proteins.

Preparation of the four SBTI's has been described earlier (5, 8, 9), and the samples used in this study are a portion of those previously prepared (9).

Composition and preparation of gels. Six gm of Cyanogum 41[2] were dissolved in 150 ml of freshly prepared gel buffer, 0.025 M glycine pH 9.2 (0.005 ionic strength) containing 8 M urea. Then 0.15 ml *N,N,N',N'*-tetramethylethylenediamine and 0.15 gm of ammonium persulfate were added in succession. The solution was stirred gently to achieve mixing without aeration and then transferred to the vertical electrophoresis apparatus (E-C Apparatus Corporation, Philadelphia, Pennsylvania) described by Raymond (12) and the slot former was inserted. Gelation required 3–15 minutes, and the gels were aged 1–3 hours before use.

Electrophoresis. After removal of excess gel and the slot former, buffer was added to the chambers. This buffer was the same as the gel buffer except that the glycine concentration was doubled (10).

Protein samples, freshly dissolved in gel buffer and with 2% sucrose added to increase the density, were inserted in the slots with a micropipette. Usually 0.5 mg (20 μl) of water-extractable, acid-precipitated, and whey proteins were inserted in the gels. Higher concentrations caused minor bands to be obscured because of streaking. For the trypsin inhibitors 0.2–0.4 mg (20 μl) of protein was used for the analysis. Bovine serum albumin stained with bromphenol blue was used as a visual marker during electrophoresis. Electrophoresis was for 1.25–1.75 hours at 15 V per centimeter (385 V potential difference, 75 mA). The buffer chambers were cooled with circulating tap water at 20°.

Protein detection. After electrophoresis the gels were removed and the proteins were stained (13) with 1% amido black 10-B in CH_3OH:H_2O:CH_3COOH (5:5:1). After staining for 5–8 minutes the gels were washed 3–4 hours with the dye solvent and finally with water for about 18 hours. The gels were photographed with a Polaroid camera in transmitted light.

[2] Mention of firm names or trade products is for identification only and does not imply endorsement by the U.S. Department of Agriculture.

RESULTS AND DISCUSSION

Figure 1a shows a typical ultracentrifuge pattern for 1.8% solution ($N \times 6.25$) of soybean whey proteins in Tris buffer (pH 8) at 0.1 ionic strength. The pattern showed only two peaks having sedimentation coefficients of 1.9 and 6.1 S. Moving-boundary electrophoresis (Tris buffer, pH 8, 0.1 ionic strength, 0°, 4.0 V per centimeter) indicated greater complexity (Fig. 1b), in agreement with earlier work (1, 3). A minimum of eight peaks designated 1–8 is suggested. Addition of crystalline SBTI to whey proteins increased the size of peak 7 (14). Soybean hemagglutinin added to the whey proteins results in a single peak in the region of peaks 2 and 3 with a skewing on the side corresponding to peak 3. Soybean hemagglutinin therefore appears to migrate as peak 2.

An even greater complexity of soybean whey proteins was indicated by electrophoresis in polyacrylamide gel with 8 M urea and 0.025 M glycine, pH 9.2 (Fig. 2). By this technique the complex mixture of proteins was resolved into at least 24 bands of varying degrees of clarity in the stained pattern. In Fig. 2 and all subsequent figures, direction of migration from the origin was toward the positive electrode. The densitometer tracing as well as electrophoretic pattern is shown in Fig. 2 because of difficulties in photographing minor bands and bands migrating in regions where streaking occurs. A relative mobility (*Rm*) scale arbitrarily set at 1 at the prominent, fast-moving band is included in Fig. 2. Advantages of this type of nomenclature system are discussed by Wake and Baldwin (15).

The band at 1 appeared to be a major component of whey proteins and, as we shall

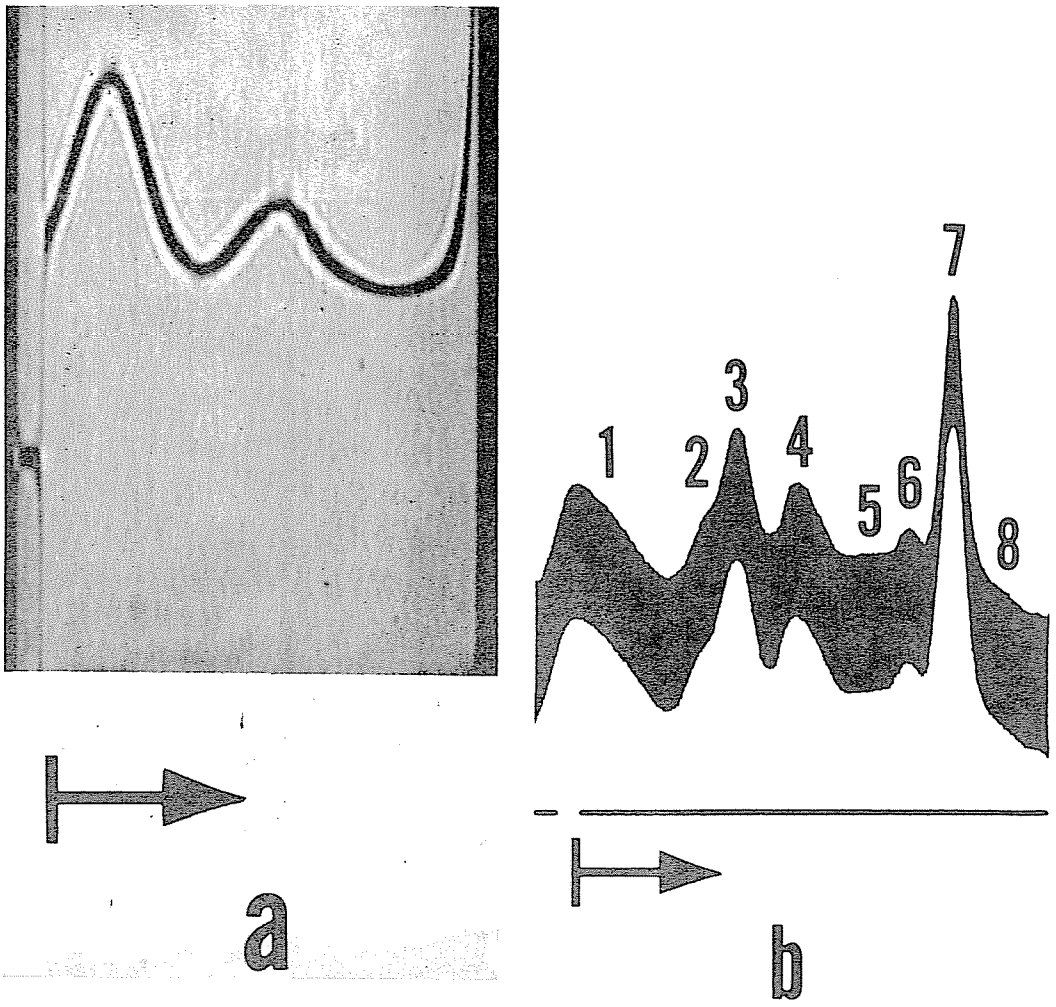

FIG. 1. Analysis of soybean whey proteins by (a) ultracentrifuge and (b) moving boundary electrophoresis (ascending pattern). Direction of migration is indicated by arrows.

show, appeared to be SBTI, which is available commercially and can be readily used as a reference standard. Seven minor bands migrating faster than the 1 band were also detectable. Five of the bands were in the 1.15–1.35 region and were only visible when the patterns were partially destained. In the completely destained pattern only two faint bands, 1.04 and 1.08, were visible, and these appeared to be the only ones migrating faster than the 1 band in Fig. 2. A number of slightly separated, but distinct, bands were observed in the region of 0.25–0.50 although they were obscured by streaking from the origin through this region. The cause of the streaking has not been determined, but the streaking was similar to the behavior of kappa casein (16, 17). However, streaking was reduced when smaller amounts of whey proteins (75–150 μg) were placed in the starting slots. Under these conditions distinct bands were observed at *Rm* values of 0.24, 0.26, and 0.28. Attempts were made to study the effect of 2-mercaptoethanol on the gel patterns because of the effect of sulfhydryl compounds on kappa casein (16) and the known ability of soybean proteins to form disulfide-linked polymers (11). Gelation of the polyacrylamide did not occur when the gel buffer contained high concentrations ($\geq$0.1 M) of 2-mercaptoethanol, possibly because the mercaptan acted as a

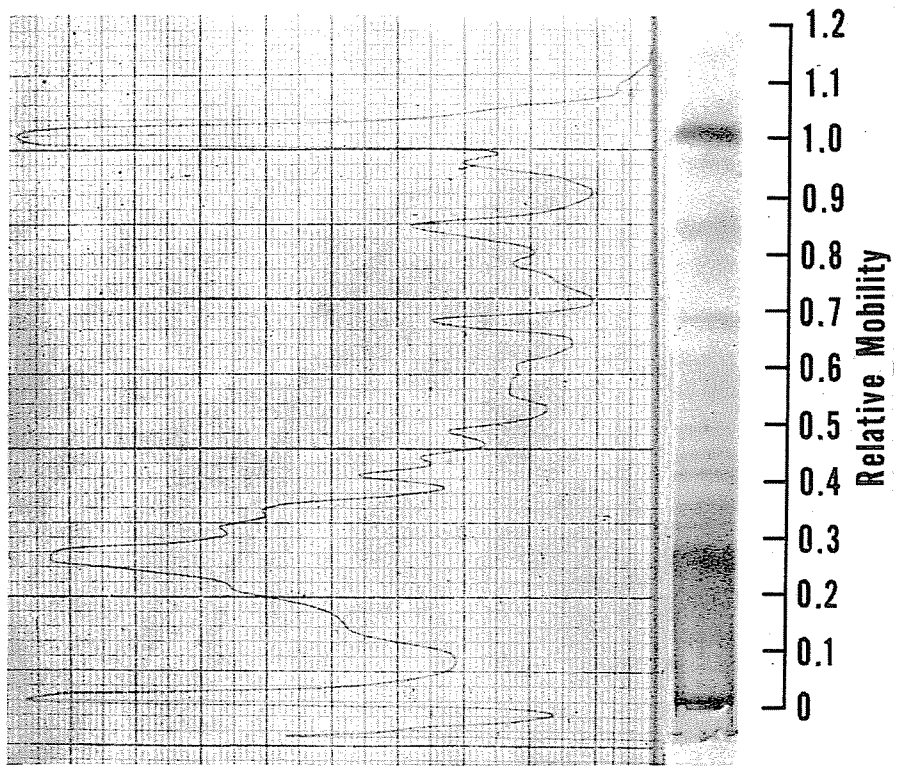

FIG. 2. Analysis of soybean whey protein by polyacrylamide-gel electrophoresis with a relative mobility scale and densitometer tracing of the gel.

chain transfer agent and kept the molecular weight of the polyacrylamide below the size required for gelation. Gelation did occur with <0.05 M 2-mercaptoethanol present in the gel buffer, but the resulting gels gave negative nitroprusside tests for mercaptan. The effect of 2-mercaptoethanol on streaking in the 0.0–0.5 *Rm* region is therefore not clear at present.

A urea concentration of 8 M was used in these studies because preliminary results showed that resolution of the whey protein bands increased with increasing concentrations of urea. It was noted, however, that urea caused aggregation of a small amount of the ordinarily soluble whey proteins. This insoluble material probably is unable to penetrate the gel and may cause the dark staining band in the starting slot.

The bulk of soybean proteins can be extracted from defatted meal with water. Acidification of a water extract of meal to pH 4.5–5 fractionates the water-extractable proteins into globulins (acid-precipitated proteins), which are insoluble, and whey proteins, which are soluble, under these conditions. Figure 3 shows the relationship between the water-extractable proteins, the two fractions obtained by acidification of a water extract, and crystalline trypsin inhibitor preparation SBTI-1 (Table I). For this experiment the gel was prepared with buffer containing 0.01 M 2-mercaptoethanol, although it is doubtful that any reducing agent remained after gelation as indicated earlier. The electrophoresis buffer contained 0.01 M 2-mercaptoethanol and was used to dissolve the samples because disulfide polymers are known to be present in the water-extractable and acid-precipitable proteins (4). The water-extractable, the acid-precipitated, and the whey proteins each had a prominent fast-moving band designated 1. The inhibitor sample contained three fast-moving bands at 1, 1.04, and 1.08, but the predominant band in SBTI-1 did not cor-

respond with the prominent 1 band in the other soybean protein samples. Instead, it corresponded to the faint 1.08 band in soybean whey, total water-extractable, and acid-precipitated soybean proteins. This dissimilarity raised a question of an artifact in the isolation of the SBTI. However, analysis of other SBTI samples indicated that SBTI-1 in Fig. 3 was atypical. Figure 4 compares various commercial samples of SBTI, some of which were crystallized five times by the procedure of Kunitz (18). All samples were stored at 2–4° after receipt or preparation. Sources and other descriptive data for the various inhibitor preparations are listed in Table I.

Figure 4 shows that all samples of SBTI yielded multiple bands. Although SBTI samples 1, 2, and 3 were from the same source, they showed a wide variation in composition. Of the samples, 3 was the newest and 1 the oldest, suggesting that aging increases the number of bands observed. This change may be coincidental, but other work in our laboratory on trypsin inhibitors indicates a decrease in trypsin inhibitor activity with age. Further work on the effect of aging of crystalline inhibitor on activity and electrophoretic behavior is in progress.

Samples 4, 5, 8, and 9, all obtained from different sources (Table I), were similar to 3, and these five preparations appeared to be the least complex of those analyzed. Sample 6, although stated to be recrystallized five times, approached the complexity of the whey protein pattern except for the absence of material in the starting slot and of streaking in the 0.00–0.50 *Rm* range. A characteristic feature of all the commercial inhibitor samples, with the exception of SBTI-1 (Fig. 4c), was the presence of a dark band at 0.94. This similarity posed the question of whether the 0.94 or the 1 band was the trypsin inhibitor. Since no attempt was made to detect trypsin inhibitor ac-

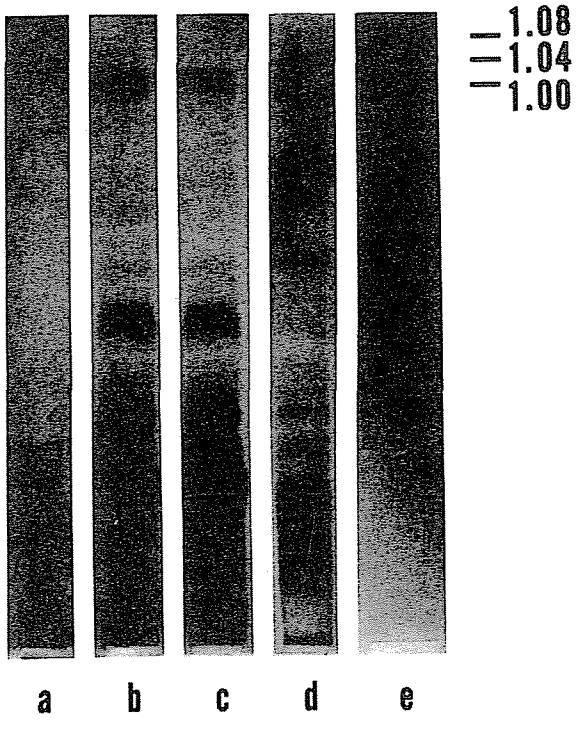

FIG. 3. Polyacrylamide-gel electrophoresis patterns of (a) bovine albumin, (b) water-extractable soybean proteins, (c) acid-precipitated soybean proteins, (d) soybean whey proteins, and (e) crystalline soybean trypsin inhibitor.

TABLE I

SOURCES, LOT NUMBERS, AND AGES OF SOYBEAN TRYPSIN INHIBITORS

SBTI Preparation	Gel pattern figure	Source	Lot number	Age[a]	Description
1	3e, 4c	Gallard Schlesinger Chemical Mfg. Co. Garden City, N. Y.	A2236	19 months	Five-times crystallized
2	4d	Gallard Schlesinger Chemical Mfg. Co. Garden City, N. Y.	A3208	12 months	Five-times crystallized
3	4e, 5g, 6g	Gallard Schlesinger Chemical Mfg. Co. Garden City, N. Y.	A4570	2 months	Five-times crystallized
4	4f	Novo Industri A/S Copenhagen, Denmark	Batch 42	Unknown	Dialyzed and lyophilized
5	4g	Nutritional Biochemical Corp. Cleveland, Ohio	2894	More than 5 years	Five-times crystallized
6	4h	Mann Research Laboratories New York, N. Y.	2414	1 week	Five-times crystallized
7	4i	Sigma Chemical Corp. St. Louis, Mo.	54B-0720	1 week	Three-times crystallized
8	4j	California Corp. for Biochemical Research Los Angeles, Calif.	43250	1 week	Three-times crystallized
9	4k	Worthington Biochemical Corp. Freehold, N. J.	SI 5490	1 week	Three-times crystallized
A_1	6e	Northern Regional Research Laboratory	6778-52-4	2 months	Prepared by column chromatography[b]
A_2	4b, 5d, 6f	Northern Regional Research Laboratory	6588-100-5	8 months	Prepared by column chromatography
B_1	6c	Northern Regional Research Laboratory	6778-52-2	2 months	Prepared by column chromatography
B_2	6d	Northern Regional Research Laboratory	6778-52-3	2 months	Prepared by column chromatography

[a] Age is from time of purchase of commercial samples until electrophoretic analysis.
[b] Method of preparation is described in Ref. 8.

tivity in the various bands of the gel patterns, an unequivocal answer cannot be given. Nevertheless, the pattern for SBTI-A_2 (Fig. 4b) strongly suggested that the 1 band is the inhibitor. SBTI-A_2 contained only a faint band in the region of 0.94 and was previously shown to be similar, if not identical, to Kunitz' crystalline inhibitor (18). Possibly the 0.94, 1.04, and 1.08 bands in the various commercial samples are genetic variants of the 1 inhibitor. The pattern obtained for SBTI-A_2 (Fig. 4b) suggests that the 1 component can be separated from the other bands, which should facilitate the task of determining the possible presence of genetic variants.

Figure 5 shows the results of mixing a laboratory-prepared and a commercial inhibitor with whey proteins and analyzing them by gel electrophoresis. SBTI-A_2 (Fig. 5d) isolated by column chromatography only had bands corresponding to those present in

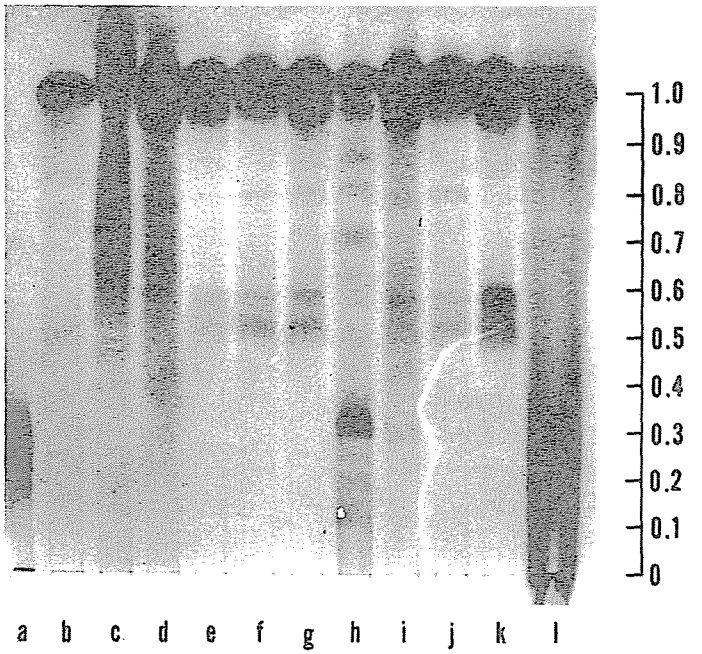

FIG. 4. Comparison of SBTI-A_2 (prepared by column chromatography), commercial samples of soybean trypsin inhibitor, and soybean whey proteins by polyacrylamide-gel electrophoresis: (a) bovine albumin, (b) SBTI-A_2, (c) SBTI-1, (d) SBTI-2, (e) SBTI-3, (f) SBTI-4, (g) SBTI-5, (h) SBTI-6, (i) SBTI-7, (j) SBTI-8, (k) SBTI-9, and (l) soybean whey protein. See Table I for identification of inhibitor preparations.

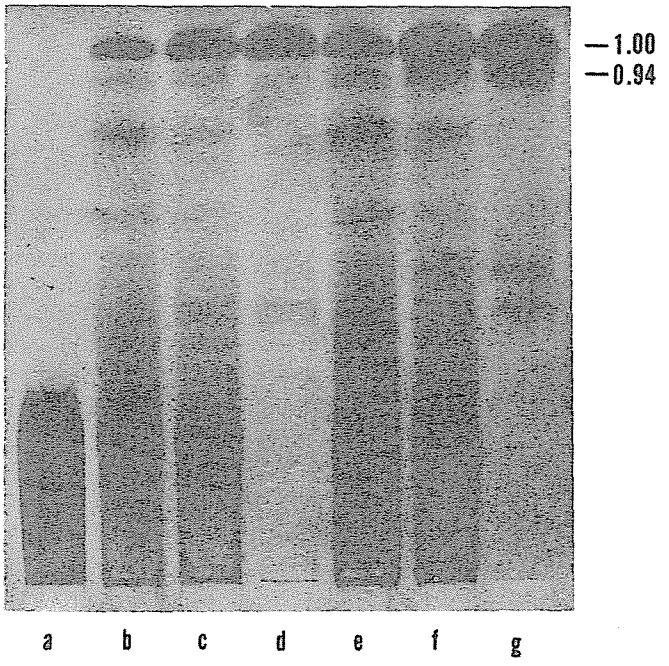

FIG. 5. Polyacrylamide-gel electrophoresis patterns of (a) bovine albumin, (b) soybean whey protein, (c) mixture of b and d, (d) SBTI-A_2, (e) soybean whey protein, (f) mixture of e and g, and (g) SBTI-3.

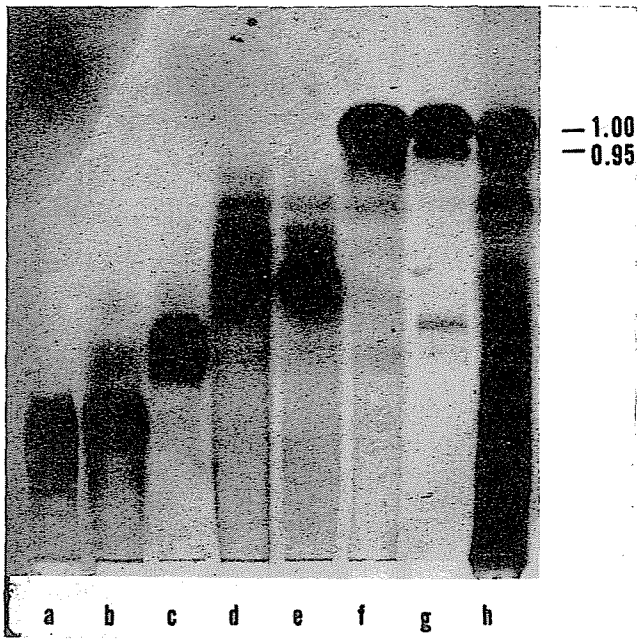

FIG. 6. Gel-electrophoresis patterns of (a) bovine albumin, (b) leading portion of DEAE column peak of SBTI-B_1, (c) SBTI-B_1, (d) SBTI-B_2, (e) SBTI-A_1, (f) SBTI-A_2, (g) SBTI-3 (commercial sample five-times crystallized), and (h) soybean whey protein.

the starting whey protein (Fig. 5b). Apparently isolation of SBTI-A_2 by column chromatography has not altered the protein. Adding SBTI-A_2 to soybean whey proteins gave a pattern (Fig. 5c) which was the sum of the patterns for the two samples when analyzed separately. The patterns in Fig. 5e and g showed that soybean whey protein (5e) and SBTI-3, a commercial SBTI crystallized five times (5g), had bands in common. Likewise, when the proteins shown as 5e and 5g were mixed (5f), no additional bands were seen. These results demonstrated that the column-isolated SBTI-A_2 and the commercial SBTI-3 five-times crystallized were similar protein mixtures except for the greatly reduced amount of band 0.94 in SBTI-A_2.

Gel patterns in Fig. 6 show the number of bands and their relative mobilities obtained for four SBTI preparations isolated by column chromatography (9). Figure 6b shows the pattern for the leading edge of fraction SBTI-B_1 as described earlier (9). Figures 6c, d, e, and f are SBTI-B_1, -B_2, -A_1, and -A_2, respectively, in the order of their elution from the DEAE-cellulose column as described by Rackis and Anderson (9). Figure 6g is SBTI-3 and Fig. 6h is a pattern of soybean whey protein. In Fig. 6 mobilities of components in the column fractions were, in general, in the order of elution from the DEAE-cellulose column, i.e., the first fractions from the column were the slower moving bands electrophoretically. This order is in agreement with Rackis *et al.* (5).

SBTI-B_1 (Fig. 6c) gave two fast-moving bands at *Rm* 0.46 and 0.54 of about equal intensity and two minor slow-moving bands. SBTI-B_2 (Fig. 6d) yielded six or more bands. SBTI-A_1 (Fig. 6e) separated into at least four bands which appeared to have the same mobility as bands in SBTI-B_2 (Fig. 6d) but in different relative concentrations. SBTI-A_2 (Fig. 6f) showed a prominent 1 band and six or more slower bands present in small amounts. The major band at 1 again corresponded to a similar band in SBTI-3 (Fig. 6g) and probably represented about 90% of the total SBTI-A_2 protein as judged by the intensity of the stained gel pattern. SBTI-3 (Fig. 6g) resolved into seven bands, but the major 1 band appeared to be only

about 75% of the total protein determined by visual observation. Figure 6h is soybean whey protein and shows the relationship of the isolated components to the total whey protein.

Cann and Goad (19) have pointed out that purified proteins may exhibit multiple bands in zone electrophoresis for three reasons: (a) heterogeneity; (b) protein isomerization; and (c) protein-solvent interaction. Ideally, the proteins in each band of a gel pattern should be isolated and then reanalyzed under conditions identical to the initial electrophoretic run in order to determine the cause of the multiple bands. However, this becomes a formidable task when 20 or more bands are observed as in the case of soybean whey proteins. Examination of patterns obtained for the various commercial trypsin inhibitors (Fig. 4) suggests that protein isomerization or protein-solvent interaction do not account for the multiple bands observed. All samples in Fig. 4 except soybean whey (Fig. 4l) were added to the gel at a level of 400 μg, yet wide variations in intensities of similar bands are observed when the various preparations are compared with each other. All inhibitors except SBTI-1 (Fig. 4c) contained the 1 band as a major component. Some samples such as Fig. 4k also showed intense bands at *Rm* values of 0.94 and 0.5–0.6, while samples such as Fig. 4f stained very lightly in these regions. The laboratory inhibitor preparation (Fig. 4b) contained only traces of protein migrating slower than the 1 band; since column chromatography virtually eliminated the 0.94 and 0.5–0.6 bands, fractionation of a protein mixture is assumed to have occurred. Furthermore, addition of inhibitor preparations containing only the 1 band or a mixture of the 1 and 0.94 bands to whey proteins failed to give rise to new bands or to intensify bands in other regions of the gel pattern (Fig. 5). The gel patterns obtained for the four inhibitor preparations obtained by DEAE-cellulose chromatography (Fig. 6) also indicate that the various bands observed in the whey protein mixture can be separated into distinct groups. Nonetheless, the possibility still exists that some of the bands observed in individual fractions such as SBTI-B_2 and SBTI-A_1 are the result of protein isomerization. Protein-solvent interaction cannot be eliminated as a source of multiple bands in these fractions but seems unlikely since this type of interaction is most pronounced at pH values acidic to the isoelectric points of proteins (19). Conditions used here are distinctly alkaline (pH 9.2) with respect to the isoelectric points of the proteins involved.

Variation of protein concentration of the four inhibitor samples did not change the gel patterns. Aging of the gels up to 24 hours likewise had no effect on the patterns obtained, which indicates that residual catalyst and free radicals, if present, had no detectable effect on the proteins.

The streaking observed in the whey protein pattern from the origin to an *Rm* value of 0.5 was decreased by reducing the protein concentration; thus protein isomerization or protein-protein interaction may have occurred in that region of the gel pattern.

Although additional work will be necessary to determine whether some of the slower migrating bands indicate heterogeneity or artifact formation, the technique described appears to be a sensitive technique for determining purity of crystalline soybean trypsin inhibitors prior to using them for experiments such as feeding studies (20) and physicochemical measurements (21, 22).

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